METHODS OF SUPPRESSING FLOWERING IN TRANSGENIC PLANTS

FIELD OF THE INVENTION

The present invention relates generally to plant molecular biology and genetic engineering and more specifically to the production of genetically modified plants in which the natural process of flowering is suppressed.

BACKGROUND INFORMATION

The ecological and economic importance of wood is difficult to overstate, with the total amount of wood in the world's forests estimated at about 1.5 Gt. Thus, wood is by far the most abundant component of the terrestrial biomass. The carbon stored in wood and humus (partially degraded wood) is important in the planetary carbon cycle, which has a significant influence on global climate. In addition, wood is a leading industrial component of the global economy. About 4% of the US gross national product has been attributed to the wood products industry in past decades.

Unfortunately, a growing population is reducing the arable land area in the United States and around the world, while the demand for wood products increases. This growing demand and limited resources have resulted in a need for greater productivity of the remaining forest lands.

The flowering process consumes 25 to 35% of the energy of a typical plant, thereby limiting wood production. Thus, for trees used for lumber or pulp production, for example, it can be advantageous to suppress flowering in order increase the yield of wood. Suppression of flowering also can be desired to eliminate the production of allergic pollen, or to prevent pollen dissemination. Unfortunately, methods of producing genetically modified plants in which flowering is suppressed without effecting other desirable traits are not currently available.

Thus, a need exists for developing genetically modified plant varieties in which the natural process of flowering is suppressed. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a transgenic plant characterized by suppressed flowering. The transgenic plant contains a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, wherein the nucleic acid molecule is heritable by progeny thereof.

The transgenic plant contains a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, where the floral organ selective regulatory element is an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element, or a *AP1* regulatory element, and wherein the nucleic acid molecule is heritable by progeny thereof.

In a transgenic plant of the invention, the floral organ selective regulatory element can be, for example, an *AGL2* regulatory element having substantially the nucleotide sequence of *Arabidopsis AGL2* promoter SEQ ID NO:1, or an active fragment thereof. A floral organ selective regulatory element useful in a transgenic plant of the invention also can be, for example, an *AGL4* regulatory element such as an *AGL4* regulatory element having substantially the nucleotide sequence of *Arabidopsis AGL4* promoter SEQ ID NO:2, or an active fragment thereof. A floral organ selective regulatory element also can be an *AGL9* regulatory element such as an *AGL9* regulatory element having substantially the nucleotide sequence of *Arabidopsis AGL9* promoter SEQ ID NO:3, or an active fragment thereof. A floral organ selective regulatory element also can be an *AP1* regulatory element such as an *AP1* regulatory element having substantially the nucleotide sequence of *Arabidopsis AP1* promoter SEQ ID NO:10, or an active fragment thereof.

DNA sequences encoding a variety of encoded cytotoxic gene products can be used to produce a transgenic plant of the invention, including DNA encoding toxic peptides such as the diphtheria toxin A chain, RNase T1, Barnase RNase, ricin toxin A chain or the herpes simplex virus thymidine kinase (tk) gene product.

The invention further relates to regenerated fertile seedlings and mature plants obtained from transgenic seed or from the vegetative reproduction of transgenic plants, and R1 and subsequent generations, produced by sexual propagation or vegetative reproduction.

The description of the invention hereafter refers to *Arabidopsis thaliana*, when necessary for the sake of example. However, it should be noted that the invention is not limited to genetic transformation of plants such as *Arabidopsis*. The method of the present invention is capable of being practiced for other plant species, including for example, other

angiosperm, and other gymnosperm forest plant species, legumes, grasses, other forage crops and the like. Particularly useful transgenic plants can be perennial woody plants such as *Eucalyptus,* cottonwood, birch, alder, Douglas fir, hemlock, pine and spruce.

The present invention also provides a tissue derived from a transgenic plant characterized by suppressed flowering and containing a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, wherein the nucleic acid molecule is heritable by progeny thereof.

The present invention further provides tissue derived from a transgenic plant characterized by suppressed flowering and containing a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, where the floral organ selective regulatory element is an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element, or an *AP1* regulatory element, wherein the nucleic acid molecule is heritable by progeny thereof. A tissue derived from a transgenic plant of the invention can be, for example, a tissue that is capable of vegetative or non-vegetative propagation, or plant cells, plant parts and seed.

The invention additionally is directed to all products derived from transgenic plants, plant cells, plant parts and seeds, which contain a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, wherein the nucleic acid molecule is heritable by progeny thereof.

The invention also is directed to all products derived from transgenic plants, plant cells, plant parts and seeds, which contain a nucleic a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, where the floral organ selective regulatory element is an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element, or an *AP1* regulatory element, wherein the nucleic acid molecule is heritable by progeny thereof.

Also provided by the present invention is a method of producing a fertile, transgenic plant characterized by suppressed flowering. The method is based upon transformation of plant material, selection, plant regeneration, and conventional or propagation breeding techniques.

The method includes the step of introducing into a plant an exogenous nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product (a peptide), wherein the nucleic acid molecule is heritable by asexual or sexually obtained progeny thereof. The method includes

the step of introducing into a plant an exogenous nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, where flowering is suppressed due to selective expression of the exogenous nucleic acid molecule and where the floral organ selective regulatory element is preferably an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element, or the *AP1* regulatory element.

The present invention also provides an isolated nucleic acid molecule including an *AGL2, AGL4* or *AGL9 or AP1* regulatory element, which confers selective expression upon an operatively linked nucleotide sequence (structural gene) in one or more floral organs of a plant.

The isolated nucleic acid molecule can further include, if desired, an operatively linked nucleotide sequence encoding a cytotoxic gene product. The encoded cytotoxic gene product can be one of a variety of cytotoxic gene products such as the peptides diphtheria toxin A chain, RNase T1, Barnase RNase, ricin toxin A chain or herpes simplex virus thymidine kinase gene product.

The present invention also provides a kit for producing a transgenic plant characterized by suppressed flowering. A kit of the invention comprises packaging containing a plant expression vector comprising a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, and instructions for transforming a susceptible plant with said vector.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a through 1e shows the *Arabidopsis AGL2* promoter SEQ ID NO:1.

Figure 2a through 2f shows the *Arabidopsis AGL4* promoter SEQ ID NO:2.

Figure 3a through 3q shows the *Arabidopsis AGL9* promoter SEQ ID NO:3.

Figure 4 shows the nucleotide (SEQ ID NO:4) and amino acid sequence (SEQ ID NO:5) of the *AGL2* cDNA and the nucleotide (SEQ ID NO:6) and amino acid sequence (SEQ ID NO:7) of the *AGL4* cDNA. The *AGL2* sequences are shown above the *AGL4* sequences.

Figure 5 shows the nucleotide (SEQ ID NO:8) and deduced amino acid sequence (SEQ ID NO:9) of the *AGL9* cDNA.

Figure 6a through 6f shows the *Arabidopsis AP1* promoter SEQ ID NO: 10.

Figure 7 shows a diagram of reporter construct POP10. The construct has 1.7 kb *AP1* promoter plus the entire coding region of *AP1* in front of promoterless GUS gene in pBI101.2

plasmid. The construct has 1.7 kb *AP1* promoter plus the entire coding region of *AP1* in front of promoterless GUS gene in pBI101.2 plasmid. The construct was first made by PCR amplification from intron 3 to the end of *AP1* gene in exon 8 (right before stop codon) using KY65 plasmid containing *AP1* genomic region as template. The HindIII site was added to the forward primer AP1HIN [5'-CAAGCTTGTACACATTTACACTCATCACAT-3'] and BamHI site was added to reverse primer AP1BAM, [5'-CGGATCCTGCGCGAAGCAGCCAAGGTTG-3'] to aid cloning (sequence in italic are restriction sites of HindIII and BamHI). The 1.7 kb amplified fragment was cloned into plasmid pBI101.2 using HindIII and BamHI sites giving construct POP9. The 3.6 kb HindIII / XbaI fragment was isolated from KY65 plasmid and cloned into POP9 construct giving POP10 construct.

Figure 8a through 8b shows the nucleotide (SEQ ID NO:11) and deduced amino acid sequence (SEQ ID NO:12) of the *AP1* cDNA.

Figure 9 shows GUS expression in 2 representative *AP1* reporter lines. GUS activity is flower specific and GUS staining pattern largely mimics *AP1* RNA accumulation pattern.

Figure 10a through 10b shows the nucleotide (SEQ ID NO:6) and amino acid sequence (SEQ ID NO:7) of the *AGL4* cDNA.

Figure 11a through 11b shows the nucleotide (SEQ ID NO:4) and amino acid sequence (SEQ ID NO:5) of the *AGL2* cDNA.

DETAILED DESCRIPTION OF THE INVENTION

Flowering is often desirable and is the natural mechanism by which flowering plants propagate. Yet for some applications, it can be desirable to suppress flower and seed production. For example, in trees grown for lumber or pulp, wood yield can be increased by suppressing flower and seed production, which normally consumes 25 to 35% of the energy of a typical plant. Where allergic pollens are a concern, non-flowering varieties are desirable to avoid pollen dissemination. Furthermore, flowering can hasten senescence; thus, non-flowering transgenic plants can have improved longevity.

The present invention provides transgenic plants characterized by suppressed flowering. In a transgenic plant of the invention, a regulatory element that directs selective expression in one or more floral organs is used to control expression of an inhibitory or cytotoxic peptide such as diphtheria toxin or ricin. The selectively expressed cytotoxic gene product destroys

floral tissue, thereby suppressing flowering, but is not expressed significantly in vegetative or other tissues and so has no deleterious effect outside the floral tissue.

A fertile transgenic plant of the invention contains a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, wherein the nucleic acid molecule is heritable by progeny thereof. A fertile transgenic plant of the invention contains a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, the floral organ selective regulatory element is an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element or an *AP1* regulatory element, wherein the nucleic acid molecule is heritable by progeny thereof.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered beneficially by the presence of heterologous DNA that was introduced into the genotype by a process of genetic engineering, or which was initially introduced into the genotype of a parent plant by such a process and is subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions. As used herein, "genotype" refers to the sum total of genetic material within a cell, either chromosomally, or extrachromosomally borne. Therefore, the term "transgenic" as used herein does not encompass the alteration of the genotype of any plant by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization or spontaneous mutation.

The term "transgenic" may be used herein to describe a plant that contains an exogenous nucleic acid molecule or chimeric nucleic acid construct, which can be derived from an orthologous or heterologous plant or can originate from an animal or virus.

The term "exogenous," as used herein in reference to a nucleic acid molecule and a transgenic plant, means a nucleic acid molecule that is not native to the plant or that is present in the genome in other than its native association. An exogenous nucleic acid molecule can have a naturally occurring or non-naturally occurring nucleotide sequence and can be orthologous or heterologous to the plant species into which it is introduced.

The term "heritable" refers to the fact that the nucleic acid molecule is capable of transmission through a complete sexual cycle of a plant, i.e., it is passed from one plant through its gametes to progeny plants in the same manner as occurs in normal plants, or the nucleic acid can be transmitted via asexual propagation of cuttings or shoots.

The term "operatively linked," as used in reference to a regulatory element and a nucleotide sequence encoding a cytotoxic gene product, means that the regulatory element is linked so that it confers regulated expression upon the operatively linked nucleotide sequence. Thus, the term "operatively linked," as used in reference to a floral organ selective regulatory element and a nucleotide sequence encoding a cytotoxic gene product, means that the floral organ selective regulatory element is linked to the nucleotide sequence encoding the cytotoxic gene product so that the expression pattern of the floral organ selective regulatory element is conferred upon the nucleotide sequence encoding the cytotoxic gene product. It is recognized that a regulatory element and a nucleotide sequence that are operatively linked have, at a minimum, all elements essential for transcription, including, for example, a TATA box.

The term "suppressed," as used herein in reference to the flowering of a transgenic plant of the invention, means a significantly diminished extent of flowering as compared to the extent of flowering in a corresponding plant lacking a nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product. Thus, the term "suppressed" is used broadly to encompass both flowering that is significantly reduced as compared to the flowering in a corresponding non-transgenic plant, and to flowering that is completely precluded. In view of the above, one skilled in the art recognizes that a transgenic plant of the invention can be completely sterile or can be characterized by reduced fertility although generally flowering is suppressed to the extent that the transgenic plant is completely sterile.

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M. O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more

preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program.

As used herein, the term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 20-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of human MCP-1.

As used herein, the term "flowering" is used broadly to refer not only to the traditional flowering of angiosperms but also to the normal reproductive development of other plants such as conifers.

It is recognized that there can be natural variation in the extent of flowering within a plant species or variety. However, a "suppression" in flowering in a transgenic plant of the invention readily can be identified by sampling a population of the corresponding plants, such as wild type plants, and determining that the normal distribution of flowering is significant diminished, on average, as compared to the normal distribution of flowering in a population of the corresponding plant species or variety that does not have a nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product. Thus, production of transgenic plants of the invention provides a means to skew the extent of normal flowering, such that flowering is

diminished, on average, at least about 1%, 2%, 5%, 10%, 30%, 50% or 100% as compared to flowering in the corresponding plant species that does not have a nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product.

As used herein, the term "cytotoxic gene product" means a gene product, usually a peptide, that inhibits the growth of, or causes the death of, the cell in which it is expressed. Preferably, a cytotoxic gene product does not result in the death of cells other than the cell in which it is expressed. Thus, expression of a cytotoxic gene product from a floral organ selective regulatory element can be used to ablate cells within one or more floral organs without disturbing neighboring cells. A variety of cytotoxic gene products useful in plants are known in the art including toxins and enzymes, for example, diphtheria toxin A chain polypeptides; RNase T1; Barnase RNase; ricin toxin A chain polypeptides; and herpes simplex virus thymidine kinase (tk) gene products. While the diphtheria toxin A chain, RNase T1 and Barnase RNase are preferred cytotoxic gene products, or multiple nucleotide sequences encoding other cytotoxic gene products, can be used with a floral organ selective regulatory element to generate a transgenic plant of the invention characterized by suppressed flowering.

Diphtheria toxin is the naturally occurring toxin of *Cornebacterium diphtheriae*, which catalyzes the ADP-ribosylation of elongation factor 2, resulting in inhibition of protein synthesis and consequent cell death (Collier, Bacteriol. Rev. 39:54-85 (1975)). A single molecule of the fully active toxin is sufficient to kill a cell (Yamaizumi et al., Cell 15:245-250 (1978)). Diphtheria toxin has two subunits: the diphtheria toxin B chain directs internalization to most eukaryotic cells through a specific membrane receptor, whereas the A chain encodes the toxic catalytic domain. The catalytic DT-A chain does not include a signal peptide and is not secreted. Further, any DT-A released from dead cells in the absence of the diphtheria toxin B chain is precluded from cell attachment. Thus, DT-A is cell autonomous and directs killing only of the cells in which it is expressed without apparent damage to neighboring cells. The DT-A expression cassette of Palmiter et al., which contains the 193 residues of the A chain engineered with a synthetic ATG and lacking the native leader sequence, is particularly useful in the transgenic plants of the invention (Palmiter et al., Cell 50:435-443 (1987); Greenfield et al., Proc. Natl. Acad. Sci., *USA* 80:6853-6857 (1983), each of which is incorporated herein by reference).

RNase T1 of *Aspergillus oryzae* and Barnase RNase of *Bacillus amylolique-faciens* also are cytotoxic gene products useful in the transgenic plants of the invention (Thorsness and Nasrallah, Methods in Cell Biology 50:439-448 (1995)). Barnase RNase may be more generally toxic to plants than RNase T1 and, thus, is preferred in the methods of the invention.

Ricin, a ribosome-inactivating protein produced by castor bean seeds, also is a cytotoxic gene product useful in a transgenic plant of the invention. The ricin toxin A chain polypeptide can be used to direct cell-specific ablation as described, for example, in Moffat et al., Development 114:681-687 (1992). Plant ribosomes are variably susceptible to the plant-derived ricin toxin. The skilled person understands that the toxicity of ricin depends is variable and should be assessed for toxicity in the plant species of interest (see Olsnes and Pihl, Molecular Action of Toxins and Viruses, pages 51-105, Amsterdam: Elsevier Biomedical Press (1982)).

The present invention relates to the use of floral organ selective regulatory elements derived from *AGL2, AGL4* or *AGL9,* which are "*AGAMOUS-LIKE*" or "*AGL*" genes. *AGAMOUS (AG)* is a floral organ identity gene, one of a related family of transcription factors that, in various combinations, specify the identity of the floral organs: the petals, sepals, stamens and carpels (Bowman et al., Devel. 112:1-20 (1991); Weigel and Meyerowitz, Cell 78:203-209 (1994); Yanofsky, Annual Rev. Plant Physiol. Mol. Biol. 46:167-188 (1995)). The AGAMOUS gene product is essential for specification of carpel and stamen identity (Bowman et al., The Plant Cell 1:37-52 (1989); Yanofsky et al., Nature 346:35-39 (1990)). Related genes have recently been identified and denoted "*AGAMOUS-LIKE*" or "*AGL*" genes (Ma et al., Genes Devel. 5:484-495 (1991); Mandel and Yanofsky, The Plant Cell 7:1763-1771 (1995), which is incorporated herein by reference).

AGL2, *AGL4* and *AGL9*, like *AGAMOUS* and other *AGL* genes, are characterized, in part, in that each is a plant MADS box gene. The plant MADS box genes generally encode proteins of about 260 amino acids including a highly conserved MADS domain of about 56 amino acids (Riechmann and Meyerowitz, Biol. Chem. 378:1079-1101 (1997), which is incorporated herein by reference). The MADS domain, which was first identified in the *Arabidopsis AGAMOUS* and *Antirrhimum majus DEFICIENS* genes, is conserved among transcription factors found in humans (serum response factor; SRF) and yeast (MCM1; Norman et al., Cell 55:989-1003 (1988); Passmore et al., J. Mol. Biol. 204:593-606 (1988), and is the most highly conserved region of the MADS domain proteins. The MADS domain

is the major determinant of sequence specific DNA-binding activity and can also perform dimerization and other accessory functions (Huang et al., The Plant Cell 8:81-94 (1996)). The MADS domain frequently resides at the amino-terminus, although some proteins contain additional residues amino-terminal to the MADS domain.

The "intervening domain" or "I-domain," located immediately C-terminal to the MADS domain, is a weakly conserved domain having a variable length of approximately 30 amino acids (Purugganan et al., Genetics 140:345-356 (1995)). In some proteins, the I-domain plays a role in the formation of DNA-binding dimers. A third domain present in plant MADS domain proteins is a moderately conserved 70 amino acid region denoted the "keratin-like domain" or "K-domain." Named for its similarity to regions of the keratin molecule, the structure of the K-domain appears capable of forming amphipathic helices and may mediate protein-protein interactions (Ma et al., Genes Devel. 5:484-495 (1991)). The most variable domain, both in sequence and in length, is the carboxy-terminal or "C-domain" of the MADS domain proteins. Dispensable for DNA binding and protein dimerization in some MADS domain proteins, the function of the C-domain remains unknown.

The amino acid sequence of *Arabidopsis AGL2*, a protein with a calculated molecular mass of about 28.5 kDa, is shown in Figures 4 and 11a through 11b. Like other AGAMOUS-LIKE proteins, *AGL2* has a highly conserved MADS domain and a K domain (Ma et al., Genes Devel. 5:484-495 (1991). RNA dot blot hybridization was used to analyze *AGL2* expression in immature seed pods, flowers, stems, and leaves. *AGL2* RNA was preferentially expressed in flowers: a strong hybridization signal was seen in flower RNA, with a diminished level seen in RNA from immature seed pods. A faint signal was also detected in leaves. To determine whether *AGL2* is expressed in an organ-specific manner, *in situ* hybridization was performed with wild type *Arabidopsis* inflorescence sections. The results showed that *AGL2* was expressed mainly in carpels and was concentrated there in the ovules. In addition, *AGL2* was expressed at a lower level in the stamens, with expression restricted to the anthers. Thus, the *AGL2* gene is selectively expressed in floral organs, with a high level of expression seen in flowers and young seed pods and a much lower level of expression seen in leaves. These results indicate that an *AGL2* regulatory element can confer floral organ selective expression upon a heterologous linked gene.

The amino acid sequence of *AGL4* is shown in Figures 4 and 10a through 10b. The encoded protein, which has a calculated molecular mass of 28.5 kDa, has the characteristic highly conserved MADS domain. RNA dot blot hybridization was used to assess *AGL4*

expression in immature seed pods, flowers, stems, and leaves. *AGL4* was highly expressed in flowers with the expression continuing at a lower level in immature seed pods. No expression was seen in the vegetative stems and leaves. These results indicate that *AGL4* is specifically expressed in flowers and that an *AGL4* regulatory element can confer floral organ selective expression upon a heterologous linked gene.

Arabidopsis AGL9 is a 251 amino acid protein having a calculated molecular mass of 29 kDa. *AGL9* has a highly conserved MADS domain, as well as a K domain (see Figure 5). The protein encoded by *Arabidopsis AGL9* has a high degree of similarity to the products of the *TM5* gene from tomato (*Lycopersicum esculentum)*; the petunia gene *FBP2,* and the *DEFH200* gene from *Antirrhinum majus,* indicating that *TM5, FBP2* and *DEFH200* are *AGL9* orthologs (Pnueli et al., Plant J. 1:255-266 (1991); Angenent et al., Plant Cell 4:983-993 (1992); and Davies et al., EMBO J. 15:4330-4343 (1996), each of which is incorporated herein by reference). Throughout the first 160 amino acids, *AGL9* shares approximately 89% amino acid identity with the FBP2, TM5 and DEFH200 gene products.

AGL9 RNA accumulates only in flowers, with RNA blot analysis showing no detectable expression in roots, stems or cauline leaves. *In situ* hybridization analyses demonstrated that *AGL9* RNA begins to accumulate after the onset of expression of the floral meristem identity genes but before the expression of the floral organ identity genes. In particular, floral meristem identity genes such as *AP1* and *CAL* are first expressed during stage 1 flower primordia, followed by *AGL2* and *AGL4*, which are first expressed throughout stage 2 flower primordia. *AGL9* is subsequently expressed late in stage 2 in a region that does not include the outer perimeter of the flower primordium. Later in flower development, *AGL9* RNA accumulates in the petal, stamen, and carpel organs. Thus, *AGL9* is specifically expressed only in floral organs, indicating that an *AGL9* regulatory element can confer floral organ selective expression upon a heterologous linked gene.

The amino acid sequence of *AP1* is shown in Figure 8a through 8b (Mandel, 1992 Nature 360:273-277). The encoded protein, which has a calculated molecular mass of 30 kDa, has the characteristic highly conserved MADS domain. The deduced *AP1* protein is similar to the snapdragon SQUAMOSA protein, sharing 68% identical amino acid residues (Huijser et al., EMBO J. 33:1239-1249; 1992). RNA blot hybridization was used to assess *AP1* expression in roots, stems, leaves, and flowers, where it was shown to be flower specific (Id., Figure 3). Subsequent RNA tissue in situ hybridizations further defined the *AP1* RNA accumulation patter where it was shown to first

be expressed in a young flower primordium (a flower meristem) when it first becomes visible on the flanks of the shoot meristem. Additional studies showed that *API* RNA accumulates in all cells of the young flower, and that in mature flowers, *API* is expressed in sepals and petals but not in stamens and carpels (Id., Fig. 4). Thus, *API* is specifically expressed in flowers and that an *API* regulatory element can confer floral organ selective expression upon a heterologous linked gene. Proof of this concept came from fusing the *API* regulatory region to the easily assayable "GUS" marker gene and the subsequent generation of transgenic plants that had stably integrated the *API*::GUS transgene into the plant nuclear genome (the POP10 construct and resulting lines)(See Figure 9).

The *API* regulatory region includes the 1.7 kb of the *API* "promoter" (the promoter is defined as the 1700 bp immediately upstream of the *API* translation initiation codon, ATG), as well as the genomic region containing all *API* intronic sequences. Both the "full length" *API* promoter (*API* promoter plus all genomic regions containing *API* intronic sequences as shown for the POP10 construct in Figure 7) and the 1700 bp *API* promoter fragment are sufficient to express foreign genes that are operably linked to it within flowers, and thus may be suitable for suppressing flowering. Smaller constructs, such as those that do not contain all of the *API* intronic sequences, may also be flower specific, and thus it is not necessary to include all of the *API* genomic sequences to achieve complete flower-specific regulation. However, the use of the "full length" *API* regulatory region may be used for optimal flower specific expression, since these sequences will drive gene expression only in flowers.

As used herein, the term "floral organ selective regulatory element" refers to a regulatory element such as a 5', 3' or intronic regulatory element that, when operatively linked to a nucleotide sequence, confers selective expression upon the operatively linked nucleotide sequence in a limited number of plant tissues, including one or more floral organs or subparts thereof. Thus, a floral organ selective regulatory element, as defined herein, confers selective expression in the petals, sepals, stamens or carpels of a plant or in some cell types within the petals, sepals, stamens or carpels, with expression low or absent in other tissues of the plant.

A floral organ selective regulatory element can confer specific expression exclusively in cells of one or more floral organ, or can confer selective expression in a limited number of plant cell types including cells of one or more floral organ. For example, an *AGL9* regulatory element, which confers specific expression in flowers, without conferring expression in vegetative tissues such as roots, stems or cauline leaves, is a floral organ selective regulatory

element as defined herein. A floral organ selective regulatory element also can be, for example, an *AGL2* regulatory element, which confers high level expression in flowers, with a minimal level of expression in leaves.

As used herein, the term "*AGL2* regulatory element" refers to a regulatory element derived from *Arabidopsis AGL2* (SEQ ID NO:5) or an ortholog of *Arabidopsis AGL2*. An *AGL2* ortholog is a MADS box gene product expressed, at least in part, in one or more floral organs of a plant and having homology to the amino acid sequence of *Arabidopsis AGL2* (SEQ ID NO:5). An *AGL2* ortholog can be, for example, a pine or rice ortholog such as PrMADS1 or OsMADS5 (Mouradov et al., Plant Physiol. 117:55-62 (1998); Kang and An, Mol. Cells 7:45-51 (1997), each of which is incorporated herein by reference) or can be another ortholog such as a *Eucalyptus* or spruce ortholog. An *AGL2* ortholog generally has at least about 80% amino acid identity with amino acids 1 to 160 of *Arabidopsis AGL2* (SEQ ID NO:5) and can have, for example, at least about 85%, 90%, or 95% amino acid identity with amino acids 1 to 160 of *Arabidopsis AGL2* (SEQ ID NO:5).

As used herein, the term "*AGL4* regulatory element" refers to a regulatory element derived from *Arabidopsis AGL4* (SEQ ID NO:7) or an ortholog of *Arabidopsis AGL4*. An *AGL4* ortholog is a MADS box gene product expressed, at least in part, in one or more floral organs of a plant and having homology to the amino acid sequence of *Arabidopsis AGL4* (SEQ ID NO:7). An *AGL4* ortholog can be, for example, a *Eucalyptus*, pine or spruce ortholog. An *AGL4* ortholog generally has at least about 80% amino acid identity with amino acids 1 to 160 of *Arabidopsis AGL4* (SEQ ID NO:7) and can have, for example, at least about 85%, 90%, or 95% amino acid identity with amino acids 1 to 160 of *Arabidopsis* AGL4 (SEQ ID NO:7).

As used herein, the term "*AGL9* regulatory element" refers to a regulatory element derived from *Arabidopsis AGL9* (SEQ ID NO:9) or an ortholog of *Arabidopsis AGL9*. An *AGL9* ortholog is a MADS box gene product expressed, at least in part, in one or more floral organs of a plant and having homology to the amino acid sequence of *Arabidopsis AGL9* (SEQ ID NO:9). An *AGL9* ortholog can be, for example, a tomato, petunia or *A. majus* ortholog such as TM5, FBP2 or DEFH200 (Pnueli et al., The Plant Cell 6:163-173 (1994); Angenent et al., Plant Cell 4:983-993 (1992); and Davies et al., EMBO J. 15:4330-4343 (1996)) or can be, for example, a *Eucalyptus*, pine or spruce ortholog. An *AGL9* ortholog generally has at least about 80% amino acid identity with amino acids 1 to 160 of

Arabidopsis AGL9 (SEQ ID NO:9) and can have, for example, at least about 85%, 90%, or 95% amino acid identity with amino acids 1 to 160 of *Arabidopsis AGL9* (SEQ ID NO:9).

As used herein the term "*AP1* regulatory element " refers to a regulatory element derived from Arabidopsis *AP1* (SEQ ID NO:10) or an ortholog of *Arabidopsis AP1*. An *AP1* ortholog is a MADS box gene product expressed, at least in part, in one or more floral organs of a plant and having homology to the amino acid sequence of *Arabidopsis AP1* (SEQ ID NO:10). An *AP1* ortholog can be, for example, a snapdragon ortholog, such as SQUAMOSA. Also, an *AP1* ortholog could be, for example, a *Eucalyptus,* pine or spruce ortholog. An *AP1* ortholog generally has at least about 75% amino acid identity with amino acids 1 to 160 of *Arabidopsis AP1* (SEQ ID NO:10) and can have, for example, at least about 85%, 90%, or 95% amino acid identity with amino acids 1 to 160 of *Arabidopsis AP1* (SEQ ID NO:10).

Preferably, an *AGL2*, *AGL4* or *AGL9* or *AP1* floral organ selective regulatory element is orthologous to the transgenic plant species into which it is introduced. An *AGL2* promoter (SEQ ID NO:1) or active fragment thereof, for example, can be introduced into an *Arabidopsis* plant to produce a transgenic *Arabidopsis* variety characterized by suppressed flowering. Similarly, a *Eucalyptus AGL2, AGL4* or *AGL9* or *AP1* floral organ selective regulatory element can be introduced into a *Eucalyptus* plant to produce a transgenic *Eucalyptus* variety characterized by suppressed flowering.

An *AGL2*, *AGL4* or *AGL9* or *AP1* floral organ selective regulatory element also can be introduced into a heterologous plant to produce a transgenic plant of the invention characterized by suppressed flowering. AGAMOUS-like gene products have been widely conserved throughout the plant kingdom; for example, AGAMOUS has been conserved in tomato (TAG1) and maize (ZAG1), indicating that orthologs of *AGAMOUS*-like genes are present in most, if not all, angiosperms (Pnueli et al., The Plant Cell 6:163-173 (1994); Schmidt et al., The Plant Cell 5:729-737 (1993)). Furthermore, it has been shown that MADS-box genes exist in gymnosperms and angiosperms as well as in ferns, the common ancestors of contemporary seed plants (Tandre et al., Plant Mol. Biol. 27:69-78 (1995); Liu and Podila, Plant Phys. 113:665 (1997); Münster et al., Proc. Natl. Acad. Sci., USA 94:2145-2420 (1997); and Mouradov et al., Plant Physiol. 117:55-62 (1998)). *AGL2, AGL4* and *AGL9* floral organ selective regulatory elements also can be conserved and can function across species boundaries to confer floral organ selective expression in heterologous plant species. Thus, an *Arabidopsis AGL2, AGL4* or *AGL9* or *AP1* floral organ selective regulatory

element, such as the *Arabidopsis AGL2*, *AGL4* or *AGL9* or *AP1* promoter SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO: 10, or an active fragment thereof, can confer floral organ selective expression upon an operatively linked nucleotide sequence encoding a cytotoxic gene product in a heterologous plant such as *Eucalyptus*, whereby the cytotoxic gene product is selectively expressed in floral tissue and flowering is suppressed.

A transgenic plant of the invention that is characterized by suppressed flowering can be one of a variety of plant species. As used herein, the term "plant" means a higher plant that generally is a vascular plant or seed plant such as an angiosperm or gymnosperm. An angiosperm is a seed-bearing plant whose seeds are borne in a mature ovary (fruit) and are divided into two broad classes based on the number of cotyledons or seed leaves that generally store or absorb food. A gymnosperm is a seed-bearing plant with seeds not enclosed in an ovary. In view of the above, the skilled person understands that the invention can be practiced, for example, with a monocotyledonous or dicotyledonous angiosperm or gymnosperm as desired.

In one embodiment, the invention provides a transgenic woody plant that is characterized by suppressed flowering. A transgenic plant of the invention can be, for example, a perennial woody plant such as a tree or shrub. For example, dicot trees such as alder, ash, basswood, beech, birch, cherry, cottonwood, elm, hickory, locust, maple, red and white oak, persimmon, sycamore, walnut, and poplar can be modified as disclosed herein to produce transgenic varieties in which flowering is suppressed. In addition, conifer woods, for example, cedar; Douglas fir; hemlock; loblolly, ponderosa, slash, sugar and western white pines; redwood; and spruce trees can be modified to produce transgenic varieties in which flowering is suppressed. The skilled person understands that the invention can be practiced with these or other shrubs or trees, especially trees useful for producing lumber, pulp or paper (Whetten and Sederoff, Forest Ecology and Management 43:301-316 (1991), which is incorporated herein by reference).

The present invention further provides tissues derived from a transgenic plant of the invention. Such tissues are derived from a transgenic plant that is characterized by suppressed flowering and that contains a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product.

As used herein, the term "tissue" means an aggregate of plant cells and intercellular material organized into a structural and functional unit. A particularly useful tissue of the

invention is a tissue that can be vegetatively or non-vegetatively propagated such that the transgenic plant from which the tissue was derived is reproduced. A tissue of the invention can be, for example, a leaf, root, stem or part thereof.

The present invention also provides an isolated nucleic acid molecule including an *AGL2*, *AGL4* or *AGL9* or *AP1* regulatory element, which confers selective expression upon an operatively linked nucleotide sequence in one or more floral organs of a plant. The isolated nucleic acid molecule can further include, if desired, an operatively linked nucleotide sequence encoding a cytotoxic gene product. The encoded cytotoxic gene product can be, for example, diphtheria toxin A chain, RNase T1, Barnase RNase, ricin toxin A chain, or the herpes simplex virus thymidine kinase gene product.

The *Arabidopsis AGL2* promoter (SEQ ID NO:1) is shown in Figure 1. An *AGL2* regulatory element, such as a 5' regulatory element or intronic regulatory element, can confer selective expression in one or more floral organs such as carpels and stamens and, thus, is a floral organ selective regulatory element as defined herein. An isolated *AGL2* floral organ selective regulatory element can have, for example, at least fifteen contiguous nucleotides of the *Arabidopsis AGL2* sequence SEQ ID NO:1. Such an isolated *AGL2* floral organ selective regulatory element can have, for example, at least 16, 18, 20, 25, 30, 40, 50, 100 or 500 contiguous nucleotides of SEQ ID NO:1 and is characterized, in part, by the ability to confer floral organ selective expression upon an operatively linked nucleotide sequence (see Example I).

The *Arabidopsis AGL4* promoter (SEQ ID NO:2) is shown in Figure 2. An *AGL4* regulatory element confers selective expression in one or more floral organs without conferring expression in vegetative tissues and, thus, is a floral organ selective regulatory element as defined herein. An isolated *AGL4* floral organ selective regulatory element can have, for example, at least fifteen contiguous nucleotides of the *Arabidopsis AGL4* sequence SEQ ID NO:2. Such an isolated *AGL4* floral organ selective regulatory element can have, for example, at least 16, 18, 20, 25, 30, 40, 50, 100 or 500 contiguous nucleotides of SEQ ID NO:2 and is characterized, in part, by the ability to confer floral organ selective expression upon an operatively linked nucleotide sequence (see Example II).

The *Arabidopsis AGL9* promoter (SEQ ID NO:3) is shown in Figure 3. An *AGL9* regulatory element, such as a 5' regulatory element or intronic regulatory element, can confer selective expression in one or more floral organs, specifically in petals, stamens and carpels, and, thus, is a floral organ selective regulatory element as defined herein. An isolated *AGL9*

floral organ selective regulatory element can have, for example, at least fifteen contiguous nucleotides of the *Arabidopsis AGL9* sequence SEQ ID NO:3. Such an isolated *AGL9* floral organ selective regulatory element can have, for example, at least 16, 18, 20, 25, 30, 40, 50, 100 or 500 contiguous nucleotides of SEQ ID NO:3 and is characterized, in part, by the ability to confer floral organ selective expression upon an operatively linked nucleotide sequence (see Example III).

The *Arabidopsis AP1* promoter (SEQ ID NO:10) is shown in Figure 6. An *AP1* regulatory element, such as a 5' regulatory element or intronic regulatory element, can confer selective expression in one or more floral organs, specifically in petals, stamens and carpels, and, thus, is a floral organ selective regulatory element as defined herein. An isolated *AP1* floral organ selective regulatory element can have, for example, at least fifteen contiguous nucleotides of the *Arabidopsis AP1* sequence SEQ ID NO:10. Such an isolated *AP1* floral organ selective regulatory element can have, for example, at least 16, 18, 20, 25, 30, 40, 50, 100 or 500 contiguous nucleotides of SEQ ID NO:10 and is characterized, in part, by the ability to confer floral organ selective expression upon an operatively linked nucleotide sequence (see Example IV).

As used herein, the term "substantially the nucleotide sequence," when used in reference to an *AGL2, AGL4* or *AGL9* or *AP1* regulatory element, means a nucleotide sequence having an identical sequence, or a nucleotide sequence having a similar, non-identical sequence that is considered to be a functionally equivalent sequence by those skilled in the art. For example, a floral organ selective regulatory element that is an *AGL2* regulatory element can have, for example, a nucleotide sequence identical to the sequence of the *Arabidopsis AGL2* promoter (SEQ ID NO:1) shown in Figure 1, or a similar, non-identical sequence that is functionally equivalent. A floral organ selective regulatory element can have, for example, one or more modifications such as nucleotide additions, deletions or substitutions relative to the *AGL2* promoter sequence shown in Figure 1, provided that the modified nucleotide sequence retains substantially the ability to confer selective expression in one or more floral organs upon an operatively linked nucleotide sequence, such as a nucleotide sequence encoding a cytotoxic gene product.

It is understood that limited modifications can be made without destroying the biological function of an *AGL2, AGL4* or *AGL9* or *AP1* regulatory element and that such limited modifications can result in floral organ selective regulatory elements that have substantially equivalent or enhanced function as compared to a wild type *AGL2, AGL4* or

AGL9 or *AP1* regulatory element. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental such as through mutation in hosts harboring the regulatory element. All such modified nucleotide sequences are included in the definition of a floral organ selective regulatory element as long as the ability to confer selective expression in one or more floral organs is substantially retained.

A floral organ selective regulatory element can be derived from a gene that is an ortholog of *Arabidopsis AGL2, AGL4* or *AGL9* or *AP1* and that is selectively expressed in one or more floral organs of the orthologous plant. An *AGL2, AGL4* or *AGL9* or *AP1* floral organ selective regulatory element can be derived, for example, from an *AGL2, AGL4* or *AGL9* or *AP1* ortholog such as a Eucalyptus, pine or spruce ortholog.

Floral organ selective regulatory elements also can be derived from a variety of other genes that are selectively expressed in one or more floral organs of a plant and can be identified and isolated using routine methodology. Differential screening strategies using, for example, RNA prepared from a floral organ and RNA prepared from non-floral material such as leaf or root tissue can be used to isolate cDNAs selectively expressed in cells of one or more floral organs; subsequently, the corresponding genes are isolated using the cDNA sequence as a probe.

Enhancer trap or gene trap strategies also can be used to identify and isolate a floral organ selective regulatory element (Sundaresan, et al., Genes Dev. 9, 1797-1810 (1995); Koncz et al., Proc. Natl. Acad. Sci. USA 86:8467-8471 (1989); Kertbundit et al., Proc. Natl. Acad. Sci. USA 88:5212-5216 (1991); Topping et al., Development 112:1009-1019 (1991), each of which is incorporated herein by reference). Enhancer trap elements include a reporter gene such as GUS with a weak or minimal promoter, while gene trap elements lack a promoter sequence, relying on transcription from a flanking chromosomal gene for reporter gene expression. Transposable elements included in the constructs mediate fusions to endogenous loci; constructs selectively expressed in one or more floral organs are identified by their pattern of expression. With the inserted element as a tag, the flanking floral organ selective regulatory element is cloned using, for example, inverse polymerase chain reaction methodology (see, for example, Aarts et al., Nature 363:715-717 (1993); see, also, Ochman et al., "Amplification of Flanking Sequences by Inverse PCR," in Innis et al. (Ed.), PCR Protocols, San Diego: Academic Press, Inc. (1990)). The Ac/Ds transposition system of Sundaresan, et al., Genes Dev. 9, 1797-1810 (1995), can be particularly useful in identifying and isolating a floral organ selective regulatory element useful in the invention.

Floral organ selective regulatory elements also can be isolated by inserting a library of random genomic DNA fragments in front of a promoterless reporter gene and screening transgenic plants transformed with the library for floral organ selective reporter gene expression. The promoterless vector pROA97, which contains the *npt* gene and the GUS gene each under the control of the minimal 35S promoter, can be useful for such screening. The genomic library can be, for example, Sau3A fragments of *Arabidopsis thaliana* genomic DNA or genomic DNA from, for example, Eucalyptus, pine or spruce (Ott et al., Mol. Gen. Genet. 223:169-179 (1990); Claes et al., The Plant Journal 1:15-26 (1991), each of which is incorporated herein by reference).

An active fragment of an *AGL2, AGL4* or *AGL9* or *AP1* promoter, which contains a floral organ selective regulatory element, can be identified by routine techniques, for example, using a reporter gene and *in situ* expression analysis. The GUS and firefly luciferase reporter genes are particularly useful for *in situ* localization of plant gene expression (Jefferson et al., EMBO J. 6:3901 (1987); Ow et al., Science 334:856 (1986), each of which is incorporated herein by reference), and promoterless vectors containing the GUS expression cassette are commercially available, for example, from Clontech (Palo Alto, CA). To identify an active fragment containing a floral organ selective regulatory element such as an *AGL2, AGL4* or *AGL9* or *AP1* regulatory element, one or more nucleotide portions of an *AGL2, AGL4* or *AGL9* or *AP1* gene can be generated using enzymatic or PCR-based methodology (Glick and Thompson (eds.), Methods in Plant Molecular Biology and Biotechnology, Boca Raton, FL: CRC Press (1993); Innis et al. (Ed.), PCR Protocols, San Diego: Academic Press, Inc. (1990)); the resulting segments are fused to a reporter gene such as GUS and analyzed as described above.

The present invention also provides a kit for producing a transgenic plant characterized by suppressed flowering. A kit of the invention comprises packaging containing a plant expression vector having a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product. The plant expression vector can include, if desired, a nucleotide sequence encoding a selectable marker or reporter gene, along with instructions to employ the vector in accord with the present method.

The term "plant expression vector," as used herein, is a self-replicating nucleic acid molecule that provides a means to transfer an exogenous nucleic acid molecule into a plant host cell and to express the molecule therein. Plant expression vectors encompass vectors

suitable for *Agrobacterium*-mediated transformation, including binary and cointegrating vectors, as well as vectors for physical transformation.

Plant expression vectors can be used for transient expression of the exogenous nucleic acid molecule, or can integrate and stably express the exogenous sequence. One skilled in the art understands that a plant expression vector can contain all the functions needed for transfer and expression of an exogenous nucleic acid molecule; alternatively, one or more functions can be supplied in *trans* as in a binary vector system for *Agrobacterium*-mediated transformation.

In addition to a floral organ selective regulatory element and a nucleotide sequence encoding a cytotoxic gene product, a plant expression vector of the invention can contain, if desired, additional elements. A binary vector for *Agrobacterium*-mediated transformation contains one or both T-DNA border repeats and can also contain, for example, one or more of the following: a broad host range replicon, an *ori* T for efficient transfer from *E. coli* to *Agrobacterium,* a bacterial selectable marker such as ampicillin and a polylinker containing multiple cloning sites.

A plant expression vector for physical transformation can have, if desired, a plant selectable marker or a reporter gene or both, in addition to a floral organ selective regulatory element in vectors such as pBR322, pUC, pGEM and M13, which are commercially available, for example, from Pharmacia (Piscataway, NJ) or Promega (Madison, WI).

A selectable marker gene or a reporter gene can facilitate the identification and selection of transformed plants, or plant cells. Both selectable marker and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising, K., et al., Ann. Rev. Genet., 22, 421-478 (1988). Selectable marker genes includes the hygromycin B phosphotransferase coding sequence, which confers resistance to hygromycin B; the aminoglycoside phosphotransferase gene of transposon Tn5 (AphII), which encodes resistance to the antibiotics kanamycin, neomycin and G418; and genes which code for resistance or tolerance to glyphosate, 1,2-dicholoropropionic acid methotrexate, imidazolinones, sulfonylureas, bromoxynil, phophononthricin and the like.

Reporter genes which encode for easily assayable marker proteins are well known in the art. IN general, a reporter gene is a gene which ins not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by

some easily detectable property, e.g., phenotypic change or enzymatic activity. Examples of such gene are provided in Weising, et al., Ann. Rev. Genet., 22, 421-478 (1988).

In plant expression vectors for physical transformation of a plant, the T-DNA borders or the *ori* T region can optionally be included but provide no advantage.

Also provided by the present invention is a method of producing a transgenic plant characterized by suppressed flowering. The method includes the step of introducing into a plant an exogenous nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product, where flowering is suppressed due to selective expression of the exogenous nucleic acid molecule and where the floral organ selective regulatory element is an *AGL2* regulatory element, an *AGL4* regulatory element or an *AGL9* regulatory element or an *AP1* regulatory element.

Methods for producing the desired recombinant nucleic acid molecule under control of an *AGL2, AGL4* or *AGL9* or *AP1* floral organ selective regulatory element and for producing a transgenic plant of the invention are well known in the art (see, generally, Sambrook et al. (eds.) Molecular Cloning: A Laboratory Manual (Second Edition, Plainview, NY: Cold Spring Harbor Laboratory Press (1989); Glick and Thompson, *supra,* 1993).

An exogenous nucleic acid molecule can be introduced into a plant using a variety of transformation methodologies including *Agrobacterium*-mediated transformation and direct gene transfer methods such as electroporation and microprojectile-mediated transformation (see, generally, Wang et al. (eds), Transformation of Plants and Soil Microorganisms, Cambridge, UK: University Press (1995), which is incorporated herein by reference).

Transformation methods based upon the soil bacterium *Agrobacterium tumefaciens* are particularly useful for introducing an exogenous nucleic acid molecule into a plant. The wild type form of *Agrobacterium* contains a Ti (tumor-inducing) plasmid that directs production of tumorigenic crown gall growth on host plants. Transfer of the tumor-inducing T-DNA region of the Ti plasmid to a plant genome requires the Ti plasmid-encoded virulence genes as well as T-DNA borders, which are a set of direct DNA repeats that delineate the region to be transferred. An *Agrobacterium*-based vector is a modified form of a Ti plasmid, in which the tumor inducing functions are replaced by the nucleic acid sequence of interest to be introduced into the plant host.

Agrobacterium-mediated transformation generally employs cointegrate vectors or, preferably, binary vector systems, in which the components of the Ti plasmid are divided between a helper vector, which resides permanently in the *Agrobacterium* host and carries the

virulence genes, and a shuttle vector, which contains the gene of interest bounded by T-DNA sequences. A variety of binary vectors are well known in the art and are commercially available, for example, from Clontech (Palo Alto, CA). Methods of coculturing *Agrobacterium* with cultured plant cells or wounded tissue such as leaf tissue, root explants, hypocotyledons, stem pieces or tubers, for example, also are well known in the art (Glick and Thompson, *supra,* 1993). Wounded cells within dicot plant tissue that have been infected by *Agrobacterium* can develop organs *de novo* when cultured under the appropriate conditions; the resulting transgenic shoots eventually give rise to transgenic plants that ectopically express a nucleic acid molecule containing a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product. *Agrobacterium* also can be used for transformation of whole plants as described in Bechtold et al., C.R. Acad. Sci. Paris, Life Sci. 316:1194-1199 (1993), which is incorporated herein by reference).

Microprojectile-mediated transformation also can be used to produce a transgenic plant containing a nucleic acid molecule including a floral organ selective regulatory element operatively linked to a nucleotide sequence encoding a cytotoxic gene product. This method, as described by Lundquist et al., U.S. Pat. No. 5,554,798, which is incorporated herein by reference), relies on microprojectiles such as gold or tungsten that are coated with the desired nucleic acid molecule by precipitation with calcium chloride, spermidine or PEG. The microprojectile particles are accelerated at high speed into an angiosperm tissue using a device such as the BIOLISTIC PD-1000 (Biorad; Hercules CA).

Microprojectile-mediated delivery or "particle bombardment" is especially useful to transform plants that are difficult to transform or regenerate using other methods. Microprojectile-mediated transformation has been used, for example, to generate a variety of transgenic plant species, including cotton, tobacco, corn, hybrid poplar and papaya (see Glick and Thompson, *supra,* 1993) as well as cereal crops such as wheat, oat, barley, sorghum and rice (Duan et al., Nature Biotech. 14:494-498 (1996); Shimamoto, Curr. Opin. Biotech. 5:158-162 (1994), each of which is incorporated herein by reference). In view of the above, the skilled artisan will recognize that *Agrobacterium*-mediated or microprojectile-mediated transformation, as disclosed herein, or other methods known in the art can be used to produce a transgenic plant of the invention characterized by suppressed flowering.

Following transformation via any method, it is necessary to identify and select those plants or cells which both contain the heterologous DNA and still retain sufficient

regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively, and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus or plant which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells or plants while simultaneously inhibiting the growth of the non-transformed cells or plants.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably, the concentration of the agent is initially such that about a 5-40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells or plants to preferentially grow and divide while inhibiting untransformed cells or plants, but not to the extent that growth of the transformed cells or plants is prevented. Once the few individual transformed cells or plants have grown sufficiently, the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells or plants habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative transformants can generally be identified as viable plants. In the case of transformation of cells, putative transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells.

Once a putative transformant is identified, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to visually inspect the plants. The plants which appear to be green, growing, and healthy are compared to a control on various levels of the selective agent. Another example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e., Southern blot or polymerase chain reaction (PCR) or the like.

EXAMPLE I

AN *AGL2* REGULATORY ELEMENT DIRECTS FLORAL ORGAN SELECTIVE EXPRESSION

This example shows that a fragment of the *Arabidopsis AGL2* promoter is sufficient to direct floral organ selective gene expression.

Agrobacterium tumefaciens strain C58 was used to transform *Arabidopsis thaliana*, ecotype Columbia. The transformation method of this example was disclosed by Bechtold et al., C. R. Acad. Sci. Paris, 316:1194-9 (1993)(incorporated by reference herein).

A BglII fragment of approximately 2.3 kb was isolated from the *Arabidopsis AGL2* promoter (SEQ ID NO:1) shown in Figure 1 using the BglII sites indicated at nucleotide 1 and nucleotide 1120. The fragment was subcloned into the BamHI site of pGEM3Z (Promega, Madison, WI). The resulting plasmid was restricted with SalI and SmaI and subcloned into the corresponding sites of the GUS expression vector pBI101.2 (CLONTECH, Palo Alto, CA) to create pKY18. Analysis of GUS expression in kanamycin resistant *Arabidopsis* lines transformed with pKY18 revealed floral specific GUS expression with no significant expression in tissues other than flowers.

These results indicate that the 2.3 kb *Arabidopsis AGL2* promoter fragment of SEQ ID NO:1 directs floral organ selective expression of a heterologous linked gene product.

EXAMPLE II

AN *AGL4* REGULATORY ELEMENT DIRECTS FLORAL ORGAN SELECTIVE EXPRESSION

This example shows that a fragment of the *Arabidopsis AGL4* promoter is sufficient to direct floral organ selective gene expression.

Agrobacterium tumefaciens strain C58 was used to transform *Arabidopsis thaliana*, ecotype Columbia. The transformation method of this example was disclosed by Bechtold et al., C. R. Acad. Sci. Paris, 316:1194-9 (1993)(incorporated by reference herein).

AGL4 promoter fragments were isolated from the promoter sequence shown in Figure 2 (SEQ ID NO:2). A 560 bp *AGL4* fragment of SEQ ID NO:2 was prepared containing the region from nucleotide -862 to nucleotide -303 using the HindIII site indicated at nucleotide -862 and an engineered BamHI site. The 560 bp fragment was subcloned into the HindIII and BamHI sites of pGEM3Z (Promega). A 270 bp *AGL4* fragment of SEQ ID NO:2 was prepared similarly using the indicated DraI site at nucleotide -573 and an engineered BamHI site at nucleotide -303 and subcloned into the HincII and BamHI sites of pGEM3Z. The 560 bp and 270 bp fragments were subsequently cloned into the GUS expression vector pBI101.1 (CLONTECH) to produce pSR34 and pSR35, respectively.

Plants were transformed with pSR34 and pSR35. GUS staining was observed in the flowers of pSR34 plants. These results demonstrate that the 560 bp fragment of the *Arabidopsis AGL4* promoter confers floral organ selective expression upon a linked gene.

EXAMPLE III

AN *AGL9* REGULATORY ELEMENT DIRECTS FLORAL ORGAN SELECTIVE EXPRESSION

This example shows that a fragment of the *Arabidopsis AGL9* promoter is sufficient to direct floral organ selective gene expression.

Agrobacterium tumefaciens strain C58 was used to transform *Arabidopsis thaliana*, ecotype Columbia. The transformation method of this example was disclosed by Bechtold et al., C. R. Acad. Sci. Paris, 316:1194-9 (1993)(incorporated by reference herein).

The entire 1755 bp *AGL9* promoter fragment shown in Figure 3 (SEQ ID NO:3) was cloned into the GUS expression vector pBI101.3 (CLONTECH) to produce pSP112. Multiple transgenic lines containing pSP112 were analyzed for GUS expression. The results

showed that GUS was expressed only in floral organs, with no expression evident in other tissues such as stem.

These results demonstrate that an *AGL9* promoter is a floral organ selective regulatory element that can confer floral organ selective expression upon an operatively linked encoded gene such as GUS.

EXAMPLE IV

AN AP1 REGULATORY ELEMENT DIRECTS FLORAL ORGAN SELECTIVE EXPRESSION

This example shows that a fragment of the *Arabidopsis AP1* promoter is sufficient to direct floral selective gene expression.

Agrobacterium tumefaciens strain C58 was used to transform *Arabidopsis thaliana*, ecotype Columbia. The transformation method of this example was disclosed by Bechtold et al., C. R. Acad. Sci. Paris, 316:1194-9 (1993)(incorporated by reference herein).

The entire 1.7 kb *AP1* promoter shown in Figure 6 (SEQ ID NO: 10) plus the entire coding region of *AP1* including introns was cloned into the GUS expression vector pBI101.2 to produce the POP10 construct (Figure 7). The construct was first made by PCR amplification from intron 3 to the end of *AP1* gene in exon 8 (right before stop codon) using KY65 plasmid containing *AP1* genomic region as template. The HindIII site was addded to the forward primer AP1HIN and BamHI site was added to reverse primer AP1BAM to aid cloning. The 1.7 kb amplified fragment was cloned into plasmid pBI101.2 using HindIII and BamHI sites giving construct POP9. The 3.6 kb HindIII / XbaI fragment was isolated from KY65 plasmid and cloned into POP9 contruct giving POP10 contruct.

Multiple transgenic lines containing the POP10 construct were analyzed for GUS expression. The results showed the GUS was expressed specifically in the young flower primordium (See Figure 9) as soon as it arises on the flanks of the shoot meristem. No GUS staining was seen in the shoot meristem, the stem, leaves, roots, or any part of the plant other than in flowers.

All journal articles, references, and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference.

It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

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